

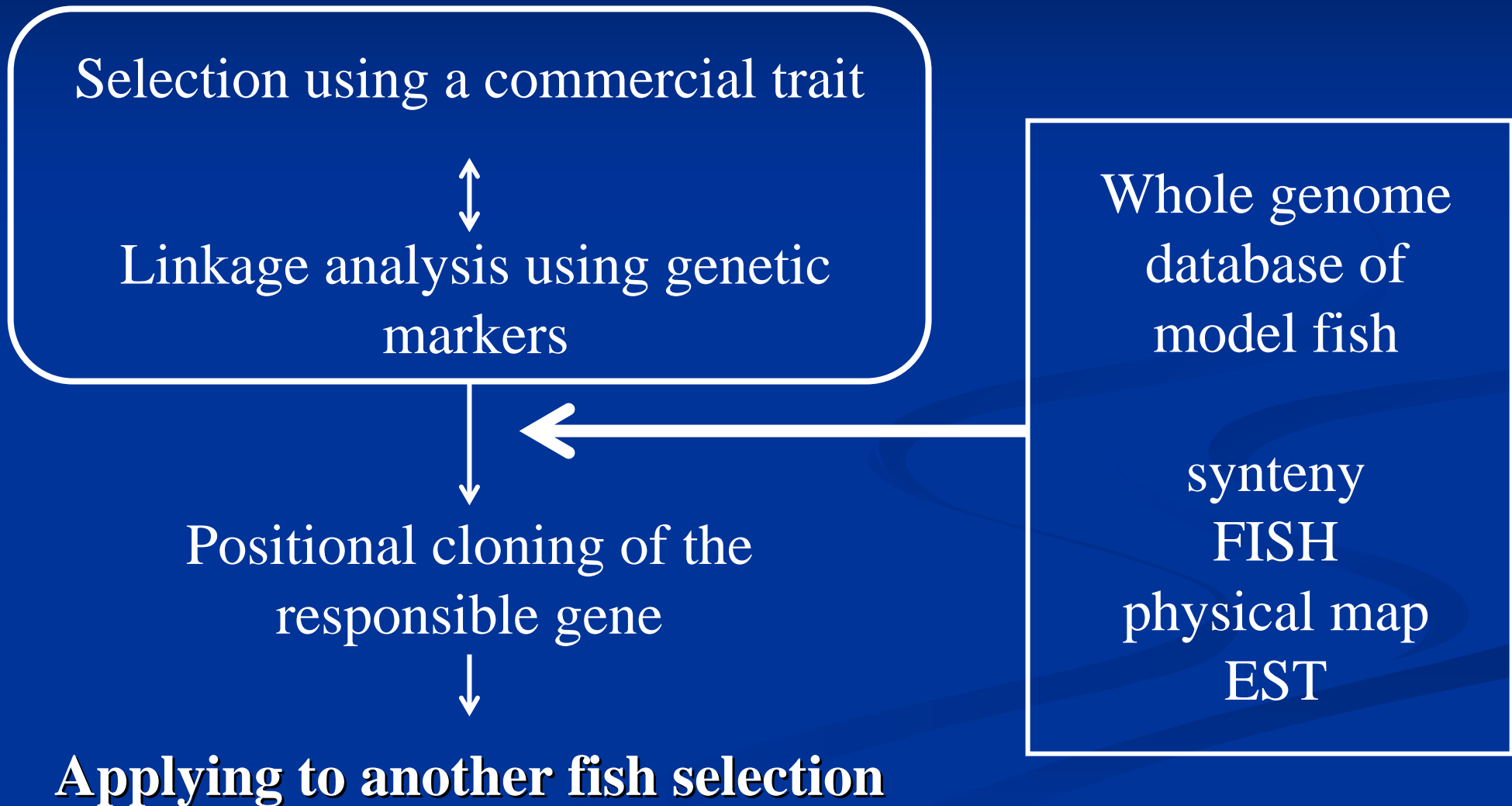
The 34th UJNR Symposium

**Construction of BAC library
from XY Japanese flounder using
frozen sperm genomic DNA**

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What can we do using the genomic information for fish breeding?



What fish is our target?



Japanese flounder (*Paralichthys olivaceus*)

- The forth amount of aquaculture production in Japan
- It is delicious, good for *sashimi* but expensive.
- The damage from an infectious disease is huge in aquaculture .
- There are several resistance groups against the disease.

Genome size of Japanese flounder

Species	haploid C-value	Mbp	No. chromosomes
Fugu	0, 4	400	44
Japanese flounder	0, 71	700	48
Medaka	0, 83	800	48
Yellowtail	0, 83	800	48
Red seabream	0, 93	900	48
Zebrafish	1, 68	1700	48
Trout	2, 07	2000	66
Human	3, 5	3000	46
			$C=0.9869 \times 10^9$ bp

Genomic breeding project (FRA) (2003-2005)

*Tokyo Univ. of
Mar. Sci. & Tech.
NRIA*

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NRIFS*

*NRIA
Hokkaido Univ.*

Recombination
map

- Microsatellite
marker

DNA resources

- Genomic library
- EST analysis

Physical map

- Chromosome
FISH

Contents

1. BAC library from frozen sperm of XY heterozygous flounder
2. Screening using the BAC library
3. Feature of MHC class Ia cluster

**#1.BAC library from frozen sperm of
XY heterozygous flounder**

How to check heterozygosity of paternal fish

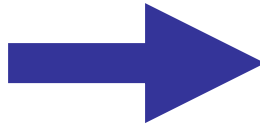
Estradiol treatment and sex ratio of offspring
from male fish TY-4

Mild $10\ \mu\text{g/l}$ of estradiol treatment



X Y genetic male

Be male



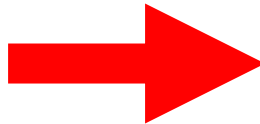
Phenotypic male

♂ 61
53%



X X genetic female

Repress sex
reversal

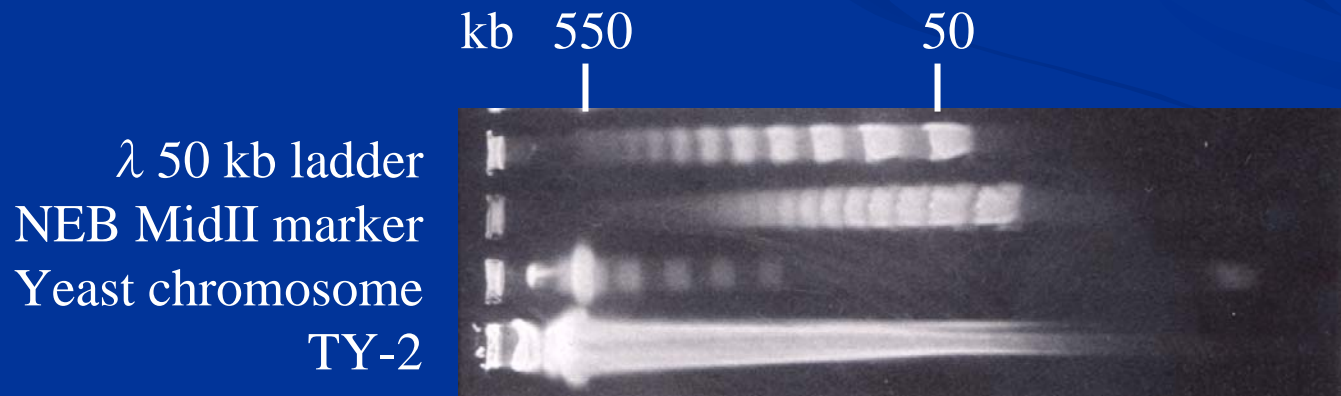


Phenotypic female

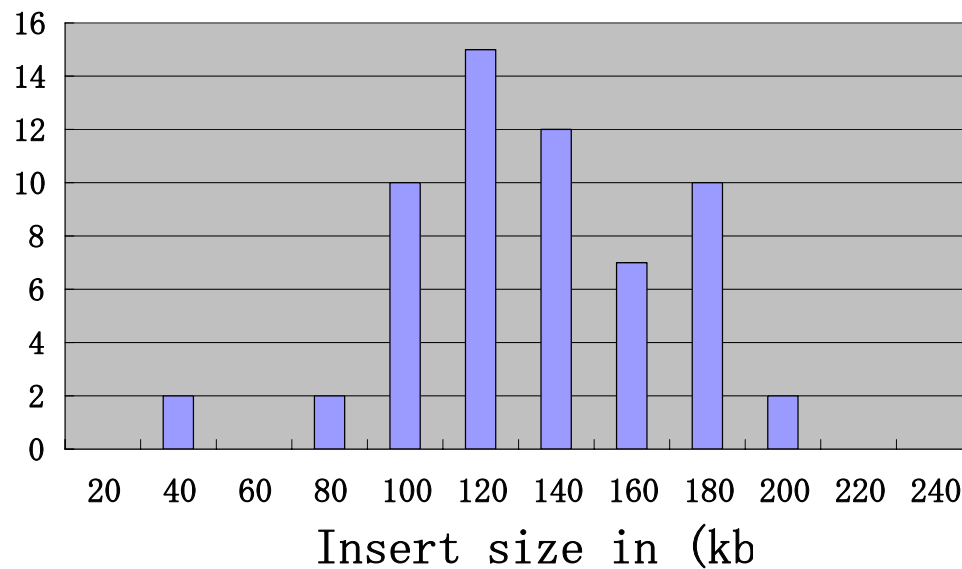
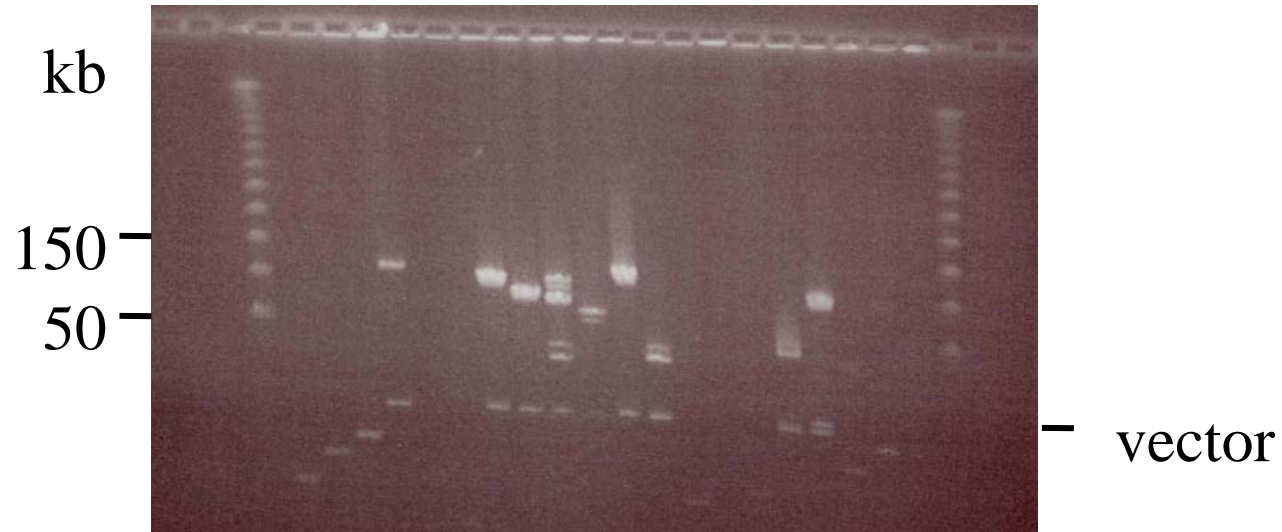
♀ 54
47%

Freezing and defrosting of sperm

- We corrected semen from five male fish, and centrifuged that to obtain a sperm pellet in June 2003.
- We frosted the sperm pellet in liquid nitrogen and kept it in a freezer at -80°C until October 2003 or December 2003 (for 4-6 months).



Insert size of BAC clones of heterozygous Japanese flounder



Ave. 140.7 kb

Size of TY-4 XY BAC library

- 110592 BAC clones were picked and arrayed in **288** of 384-well microtiter plates.
- An average insert size of **140.7 kb** was obtained by the present analysis.
- The calculations predicted a **22.2-fold** (700 Mbps) coverage of the Japanese flounder genome.

Summary #1

1. **Frozen sperm** is useful in constructing BAC and library. It facilitates the preparation of a high-molecular DNA sample, and the construction of genomic libraries.
2. We have produced a **heterozygous (XY)** genome resource of Japanese flounder as a **BAC library**.

#2. Screening using the BAC library

Objectives

- Evaluate the screening efficiency of the BAC library

What are our targets?

- **Major histocompatibility complex (MHC) cluster**, which is supposed to be related with the resistance of disease.
- **The 24 recombination linkage markers**, which will correspond to each chromosomes.

MHC (Major histocompatibility complex)

- ◆ MHC class I (I a, I b)

 - α chain & β_2 -microglobulin

 - Polymorphic domain: α_1 & α_2

 - Present antigen to cytotoxic T-cell (CD8+)

- ◆ MHC class II (II α , II β)

 - α chain & β chain

 - Polymorphic domain: α_1 & β_1

 - Present antigen to helper T-cell (CD4+)

MHC(HLA) gene cluster

- There are many genes supposed to be related with an immune system.
- The gene density in the cluster is very high (16-18 kbp /gene).
- About 20 genes each 400kbp are found in this gene cluster in model fishes.

MHC genes reported from EST analysis of Japanese flounder

Class Ia: Paol-UA1, -UA2, -UA3, -UA4, -UA5

In all tissues. Present antigen to cytotoxic T-cell

Class Ib: Paol-UB1

In lymphoid organs, gill, intestine or liver.

Present antigen to NK cell (?)

Class II α : Paol-D(01)A, -D(02)A

In all tissues. Present antigen to helper T-cell

Class II β : Paol-D(01)B, -D(02)B

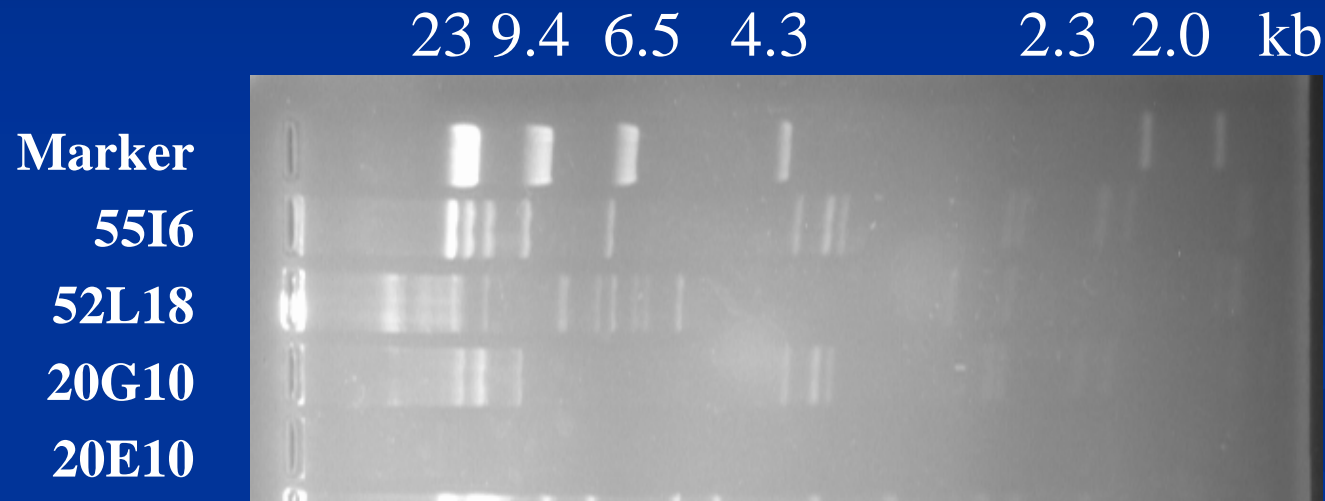
In all tissues. Present antigen to helper T-cell

Results of screening for MHC genes

MHC	Clone number		
Class Ia	20G10	52L18	55I6
Class Ib	59C10	83O20	
Class II α	15P9	56M13	
Class II β	15P9	56M13	

From 1/3 plates of library (5.9-folds coverage)

Three positive clones including MHC class Ia



EcoRI digest pattern

$52L18 \neq 55I6 > 20G10$

Check sequence of MHC class Ia

The PCR fragments amplified from the positive clones

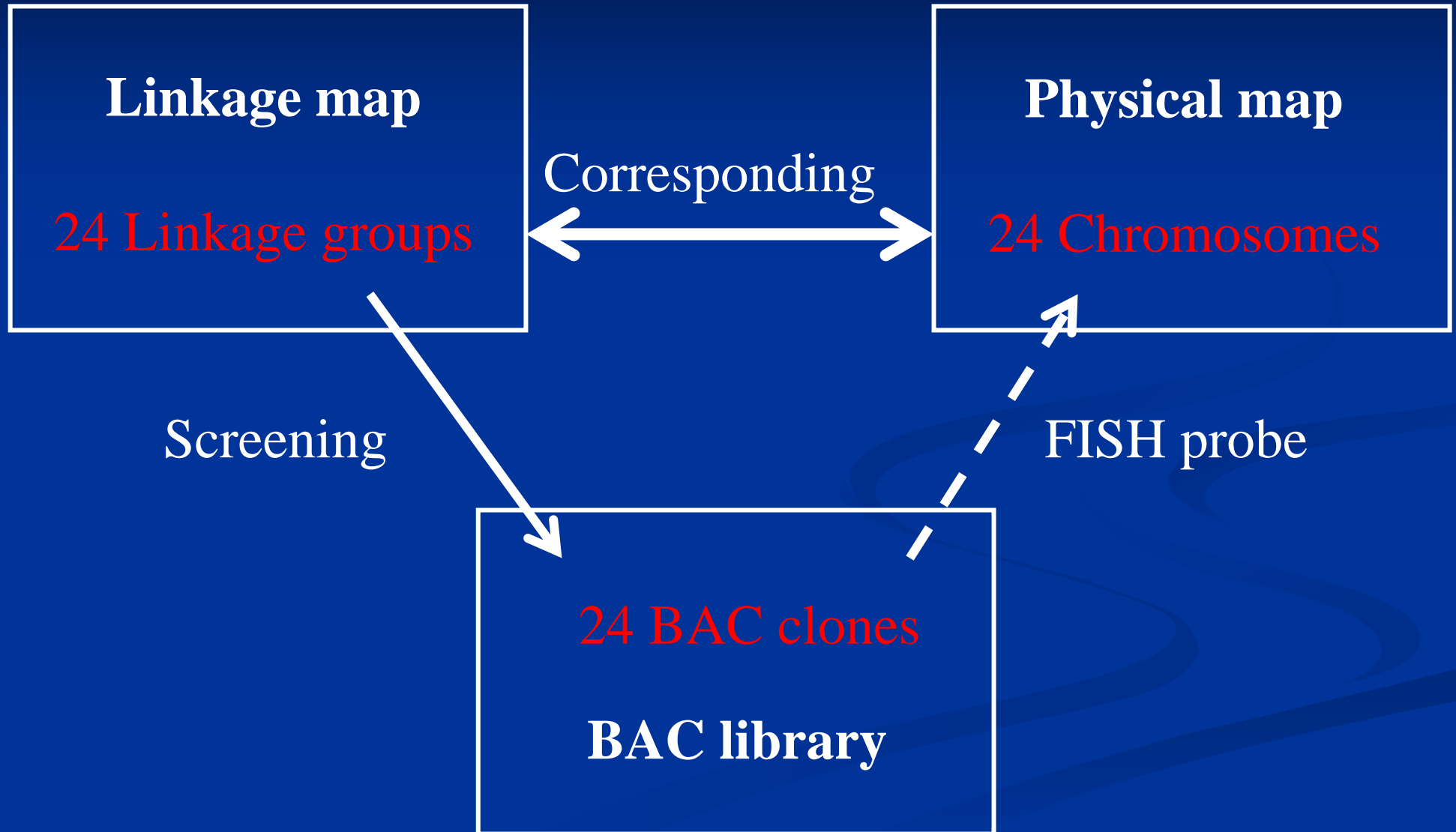
■52L18-204bp

GCGAGCTGAAACACACAGTCGTACCTCCTCCAGTCTTCAGGTGGGATT
GATGAAACCTGCAGGTCAG**T**GCTCATCTGGAAGGTCCCGTCATGGTTG
GGGAGGACCTCTCCGACGTACACGTCCTCATGAAGCTCCTCTCCGTCT
TTCCTCCAGAACAACATGGCTGAGTCGGGGTAGAAACCTGTAGCGTG
GCAGCTGACTGGA

■55I6-204bp

GCGAGCTGAAACACACAGTCGTACCTCCTCCAGTCTTCAGGTGGGATT
GATGAAACCTGCAGGTCAG**C**GCTCATCTGGAAGGTCCCGTCATGGTTG
GGGAGGACCTCTCCGACGTACACGTCCTCATGAAGCTCCTCTCCGTCT
TTCCTCCAGAACAACATGGCTGAGTCGGGGTAGAAACCTGTAGCGTG
GCAGCTGACTGGA

Correspond linkage groups to chromosomes



Summary #2

1. We screened two or three positive clones for **all four-subclass of MHC genes** from the BAC library of Japanese flounder.
2. The PCR fragments of 52L18 and 55I6 clones indicate the sequences of MHC **class Ia** gene.
3. We have screened **24 markers of each linkage groups** from the library of frozen sperm, it shows that freezing does not create any bias in the library.

#3. Feature of MHC class Ia cluster

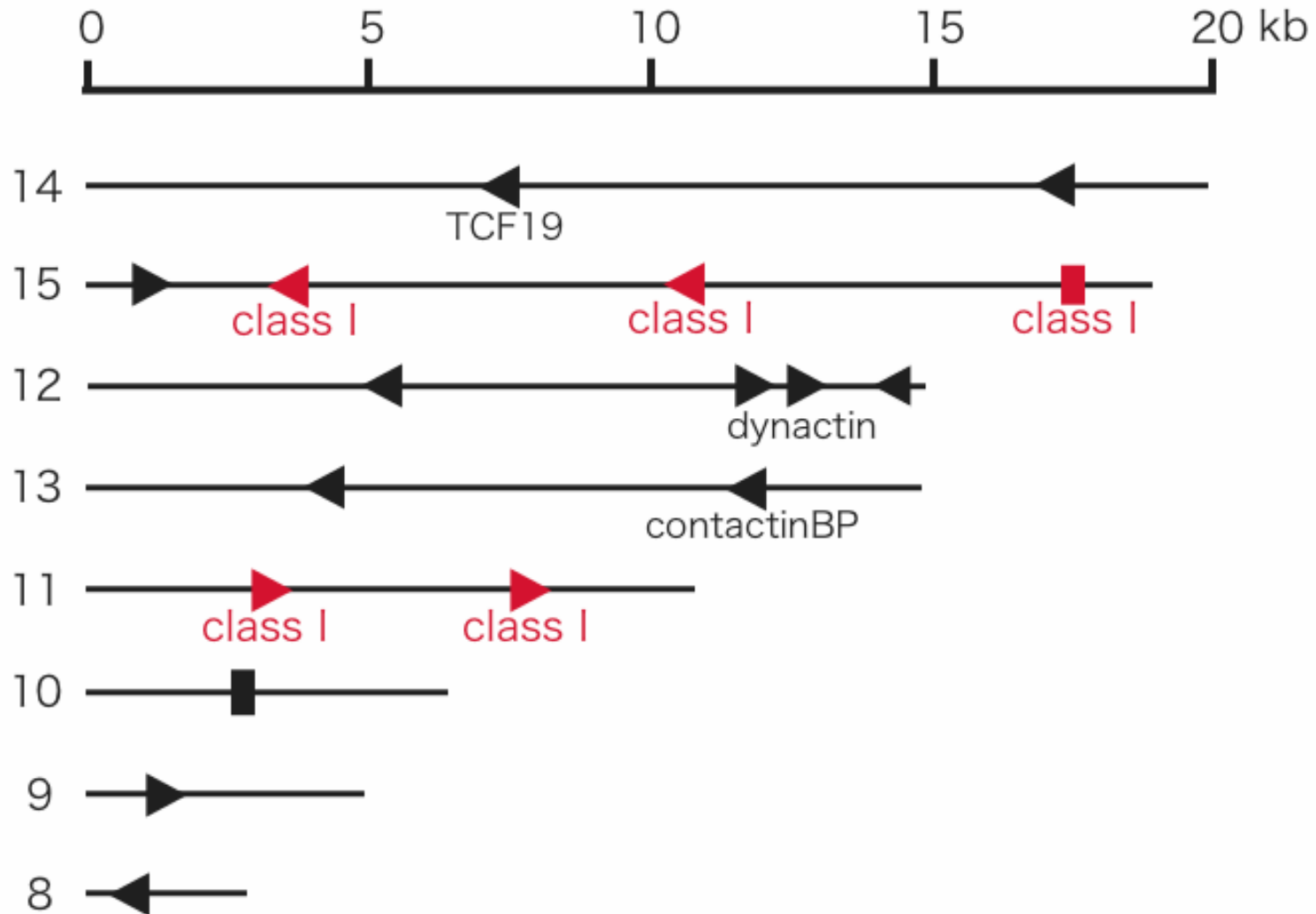
Objectives

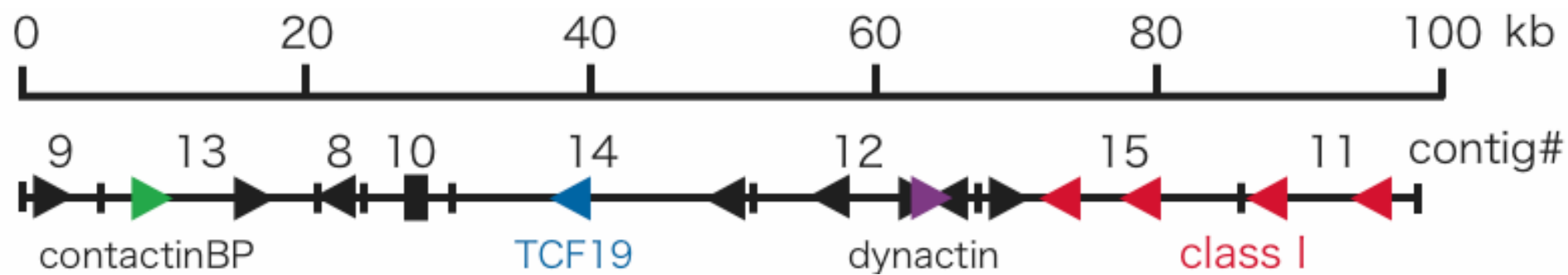
- Analyze the synteny in MHC cluster between the model fish and Japanese flounder
- Develop the linkage marker of MHC cluster, and map in the linkage group.

Presumptive genes in eight contigs of 52L18

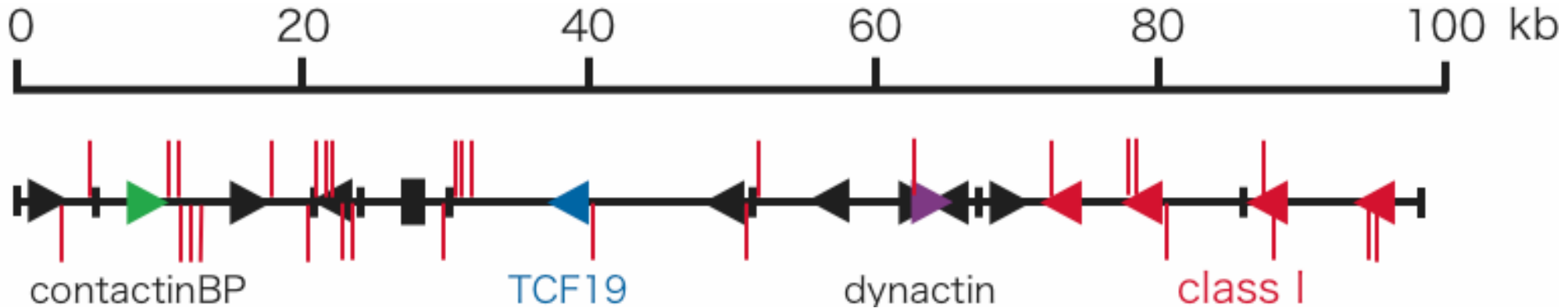
- MHC class Ia—UA4 and UA5
- TCF19
- Contactin-binding protein
- Dynactin
- Hepatitis C virus genome polyprotein
- Regulating synaptic membrane exocytosis1
- VHSV-induced protein(rainbow trout)
- Metallothionein-like

ORF prediction by GENESCAN and BLAST search





Position of MS(CA)-repeat over eight times



	Number of MS	Number of genes	Density of genes
flounder	30/95 kb (3.2 kb/MS)	8/95 kb	11.9 kb/gene
medaka	69/425 kb (6.2 kb/MS)	23/425 kb	18.5 kb/gene

Gene density in MHC of human 16kb (224 loci/3.6 Mb)

Summary #3

1. There is a **four-tandem-repeat** of MHC class Ia gene in a BAC clone.
2. The class Ia region of the flounder indicates the **high density** of microsatellite as much as that of medaka.

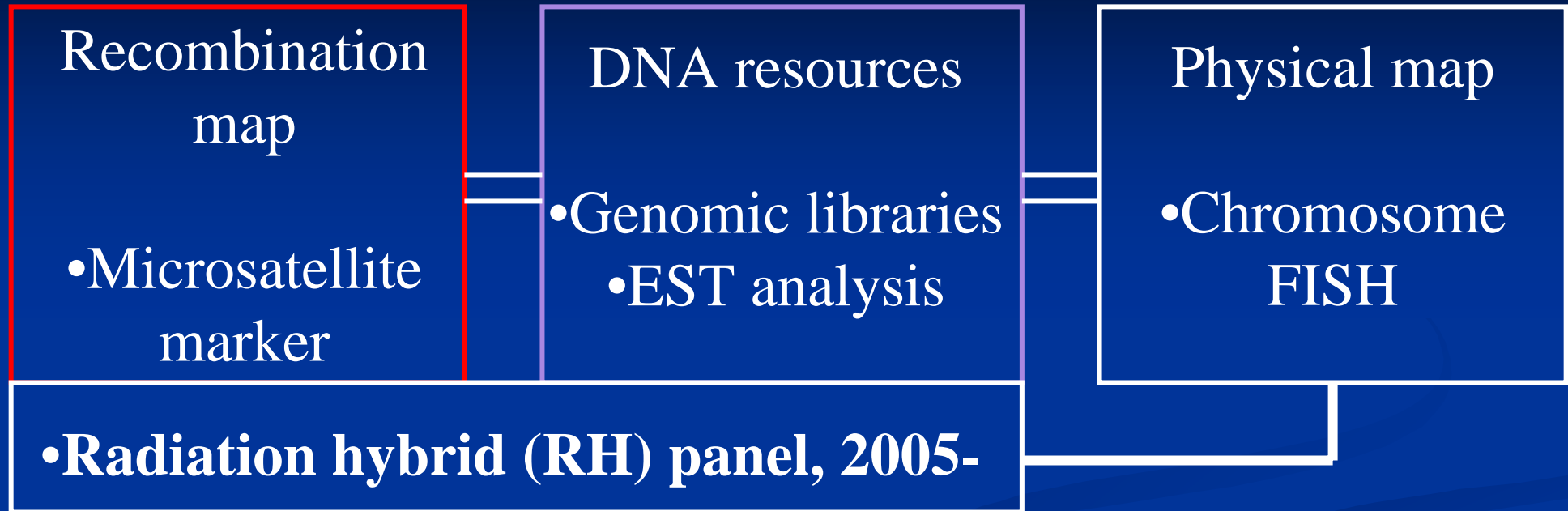
Total Summary

- **Frozen sperm** is useful for preparation of a high-molecular DNA sample, and construction of genomic (BAC, cosmid) library.
- Screening data indicates that freezing does **not create any bias** in BAC library.

In Future

Integrate genomic information using
a radiation hybrid (RH) panel(map).

Genomic breeding project (FRA), 2003-2005



RH map (Integration of MS, EST, SNP, BAC and etc.)

- access to genomic structural information of other organisms
- get a clue of positional cloning or marker development
- will be a powerful tool for proceeding of genomic breeding

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